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Genetic Basis of Attenuation of Dengue Virus Type 4 Small Plaque Mutants with Restricted Replication in Suckling Mice and in SCID Mice Transplanted with Human Liver Cells

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Mutations that restrict replication of dengue virus have been sought for the generation of recombinant live-attenuated dengue virus vaccines. Dengue virus type 4 (DEN4) was previously grown in Vero cells in the presence of 5-fluorouracil, and the characterization of 1248 mutagenized, Vero cell passaged clones identified 20 temperature-sensitive (*ts*) mutant viruses that were attenuated (*att*) in suckling mouse brain (J. E. Blaney, Jr., D. H. Johnson, C. Y. Firestone, C. T. Hanson, B. R. Murphy, and S. S. Whitehead, 2001, *J. Virol.* 75(20), 9731–9740). The present investigation has extended these studies by identifying an additional 22 DEN4 mutant viruses which have a small plaque size (*sp*) phenotype in Vero cells and/or the liver cell line, HuH-7. Five mutant viruses have a *sp* phenotype in both Vero and HuH-7 cells, three of which are also *ts*. Seventeen mutant viruses have a *sp* phenotype in only HuH-7 cells, 13 of which are also *ts*. Each of the *sp* viruses was growth restricted in the suckling mouse brain, exhibiting a wide range of reduction in replication (9- to 100,000-fold). Complete nucleotide sequence was determined for the 22 DEN4 *sp* mutant viruses, and nucleotide substitutions were found in the 3′-untranslated region (UTR) as well as in all coding regions except NS4A. Identical mutations have been identified in multiple virus clones, suggesting that they may be involved in the adaptation of DEN4 virus to efficient growth in Vero cells. Six of the 22 *sp* 5-FU mutant viruses lacked coding mutations in the structural genes, and 17 recombinant DEN4 viruses were generated which separately encoded each of the mutations observed in these six *sp* viruses. Analysis of the recombinant DEN4 viruses defined the genetic basis of the *sp*, *ts*, and *att* phenotypes observed in the six *sp* viruses. Mutations in NS1, NS3, and the 3′-UTR were found to confer a greater than 100-fold, 10,000-fold, and 1000-fold reduction in replication of rDEN4 virus in SCID mice transplanted with HuH-7 cells, respectively, which serves as a novel small animal model for DEN4 infection.

Key Words: Dengue virus; vaccine; mutagenesis; small-plaque; temperature-sensitive; attenuation.

INTRODUCTION

The dengue (DEN) viruses (serotypes 1 to 4), members of the *Flavivirus* genus, contain a single-stranded positive-sense RNA genome of approximately 10,600 nucleotides (nt) (Monath and Heinz, 1996). The genome organization of DEN viruses is 5′-UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-UTR-3′ (UTR, untranslated region; C, capsid; prM, membrane precursor; E, envelope; NS, nonstructural) (Chang, 1997; Rice, 1996). A single viral polypeptide is cotranslationally processed by viral and cellular proteases generating three structural proteins (C, M, and E) and seven NS proteins.

The DEN viruses are transmitted to humans by *Aedes* mosquitoes, which inhabit most of the tropical and semitropical regions of the world. DEN viruses cause more disease and death of humans than any other arbovirus, and more than 2.5 billion people live in regions with endemic dengue infection (Gubler, 1998). Annually, there are an estimated 50–100 million cases of dengue fever (DF) and 500,000 cases of the more severe and potentially lethal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Gubler and Meltzer, 1999). Dengue fever is an acute infection characterized by fever, retro-orbital headache, myalgia, and rash. At the time of defervescence during DF, a more severe complication of DEN virus infection, DHF/DSS, may occur, which is characterized by a second febrile period, hemorrhagic manifestations, hepatomegaly, thrombocytopenia, and hemoconcentration, which may lead to potentially life-threatening shock (Gubler, 1998).

The sites of DEN virus replication in humans and their importance and relationship to the pathogenesis of DF and DHF/DSS are still incompletely understood (Innis, 1995). In addition to replication in lymphoid cells, it has become evident that the liver is involved in DEN infection of humans. Transient elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are observed in the majority of DEN virus-infected patients and hepatomegaly is observed in some patients (Kalayanarooj *et al.,* 1997; Kuo *et al.,* 1992; Mo-

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han *et al.,* 2000; Wahid *et al.,* 2000). DEN virus antigen-positive hepatocytes are seen surrounding areas of necrosis in the liver of fatal cases (Couvelard *et al.,* 1999; Huerre *et al.,* 2001), from which dengue virus sequences were identified using RT-PCR (Rosen *et al.,* 1999). Of potential importance to the etiology of severe dengue virus infection, three studies have demonstrated that the mean levels of serum ALT and AST were significantly increased in patients with DHF/DSS than those with DF (Kalayanarooj *et al.,* 1997; Mohan *et al.,* 2000; Wahid *et al.,* 2000). As expected, elevation of serum liver enzymes has previously been observed in clinical trials of DEN virus vaccine candidates (Durbin *et al.,* 2001; Eckels *et al.,* 1984; Edelman *et al.,* 1994; Kanesa-thasan *et al.,* 2001; Vaughn *et al.,* 1996).

Based on the increasing disease burden associated with DEN virus infection over the past several decades, a vaccine that confers protection against the four dengue virus serotypes is needed, but none is presently licensed. Because of the increased risk for severe DHF/DSS associated with secondary infection with a heterologous DEN virus serotype (Burke *et al.,* 1988; Halstead *et al.,* 1977; Thein *et al.,* 1997), an effective vaccine must confer simultaneous protection against each of the four DEN virus serotypes. Several approaches are presently being pursued to develop a tetravalent vaccine against the dengue viruses (Bancroft *et al.,* 1984; Bhamarapravati and Sutee, 2000; Butrapet *et al.,* 2000; Guirakhoo *et al.,* 2000, 2001; Huang *et al.,* 2000; Kanesa-thasan *et al.,* 2001). One such approach, a live-attenuated DEN4 vaccine candidate, termed 2AΔ30, was both attenuated and immunogenic in a cohort of 20 volunteers (Durbin *et al.,* 2001). The recombinant 2AΔ30 virus contains a 30-nt deletion in the 3′UTR which removes nucleotides 10,478–10,507 and was found to produce a low or undetectable level of viremia in vaccinees at a dose of 10^5 PFU/vaccinee. An asymptomatic rash was reported in 50% of volunteers, and the only laboratory abnormality observed was an asymptomatic, transient rise in the serum ALT level in 5 of the 20 vaccinees. All 2AΔ30 vaccinees developed serum-neutralizing antibodies against DEN4 virus (mean titer: 1:580), and 2AΔ30 was not transmitted to mosquitoes that fed experimentally on vaccinees (Troyer *et al.,* 2001). Because of the desirable properties conferred by the Δ30 mutation, chimeric vaccine candidates are being constructed which contain the structural genes of DEN virus type 1, 2, and 3, in the attenuated DEN4 background bearing the genetically stable Δ30 mutation. Attenuating mutations outside of the structural genes are particularly attractive for inclusion in antigenic chimeric vaccine candidates because they will not affect the infectivity or immunogenicity conferred by the major mediator of humoral immunity to DEN viruses, the envelope protein.

The presence of rash and elevated ALT levels suggests that the 2AΔ30 vaccine candidate may be slightly underattenuated in humans. Similarly, many previous attempts to develop live-attenuated dengue virus vaccines have yielded vaccine candidates that were either over- or underattenuated in humans, some of which also induced elevation of serum ALT and AST levels (Bhamarapravati and Yoksan, 1997; Eckels *et al.,* 1984; Innis *et al.,* 1988; Kanesa-thasan *et al.,* 2001; McKee *et al.,* 1987). Therefore, we are developing a menu of point mutations conferring temperature-sensitive (*ts*), small-plaque (*sp*), and attenuation (*att*) phenotypes capable of attenuating DEN4 viruses to a varying degree (Blaney *et al.,* 2001; Hanley *et al.,* 2002). We have previously described 20 mutant viruses that exhibit a *ts*, but not *sp*, phenotype in Vero cells or HuH-7 liver cells and that show attenuated replication in mouse brain. Addition of such mutations to 2AΔ30 or to other novel DEN vaccine candidates should yield vaccine candidates that exhibit a more satisfactory balance between attenuation and immunogenicity.

In the present study, we have extended our analysis of the panel of 1248 DEN4 virus clones previously generated by mutagenesis with 5-fluorouracil (5-FU) (Blaney *et al.,* 2001), by identifying a set of 22 *sp* mutant viruses, some of which also have a *ts* phenotype. Small plaque mutant viruses were sought since such viruses are often attenuated in humans (Bhamarapravati and Yoksan, 1997; Butrapet *et al.,* 2000; Crowe *et al.,* 1994a,b; Eckels *et al.,* 1980; Innis *et al.,* 1988; Murphy and Chanock, 2001; Takemoto, 1966). Because natural infection with dengue viruses and vaccination with 2AΔ30 may be associated with liver toxicity in humans, we identified mutant viruses with restricted replication in human liver cells. Accordingly, viruses were screened for plaque size and temperature sensitivity in the human hepatoma cell line, HuH-7, as well as in Vero cells. Here we describe the *in vitro* ts phenotype, nucleotide sequence, and growth properties in suckling mice of 22 *sp* DEN4 mutant virus clones. Six *sp* 5-FU mutant viruses were found to contain coding mutations in only the NS genes and/or nucleotide substitutions in the 3′-UTR, which would facilitate the generation of antigenic chimeric viruses. The genetic basis of the observed *sp*, *ts*, and *att* phenotypes was identified for these six viruses using reverse genetics to generate recombinant DEN4 (rDEN4) viruses containing individual mutations identified in the panel of *sp* DEN4 mutant viruses. Finally, these six 5-FU mutant DEN4 viruses and corresponding rDEN4 mutant viruses were evaluated for replication in a novel small animal model for DEN4 virus replication, SCID mice transplanted with HuH-7 cells (SCID-HuH-7). These studies begin to address the utility of examining DEN virus infection in SCID mouse-xenograft models for vaccine development (An *et al.,* 1999; Lin *et al.,* 1998).

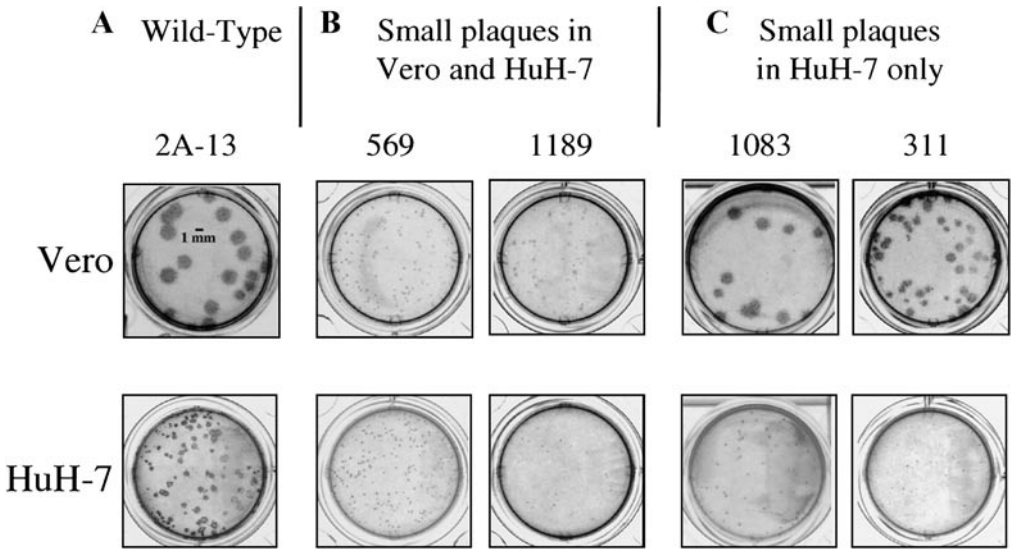

FIG. 1. Small-plaque phenotype of 5-FU mutant DEN4 viruses in Vero or HuH-7 cells. Serial 10-fold dilutions of wild-type DEN4 2A-13 (A), 5-FU mutant viruses 569 and 1189 (B), and 5-FU mutant viruses 1083 and 311 (C) were inoculated onto confluent Vero and HuH-7 cell monolayers in 24-well plates. Following incubation at 35°C for 5 days, plaques were visualized by immunoperoxidase staining. Viruses with a plaque size that was ≤1 mm in Vero cells or ≤0.4 mm in HuH-7 cells (approximately ≤50% the size of wild-type DEN4 2A-13) were designated as having the small-plaque (*sp*) phenotype. Mutant viruses 569 and 1189 (B) were *sp* in both Vero and HuH-7 cells, and 311 and 1083 (C) were *sp* in only HuH-7 cells.

RESULTS

Identification of DEN4 5-fluorouracil mutant viruses with a *sp* phenotype

The generation of a panel of 1248 virus clones from a wild-type DEN4 2A virus suspension mutagenized by 5-FU has been described previously (Blaney *et al.,* 2001). In the present study 22 mutant viruses with a *sp* phenotype were identified. The plaque size of representative mutant viruses is illustrated in Fig. 1. The plaque size of DEN4 2A-13 virus (a parallel-passaged virus with a wild-type phenotype derived from control cultures not treated with 5-FU) was consistently smaller in HuH-7 cells than that observed in Vero cells (Fig. 1A). Mutant viruses 569 and 1189 (Fig. 1B) were *sp* in both Vero and HuH-7 cells. In contrast, 5-FU mutant virus clones 311 and 1083 (Fig. 1C) were *sp* in only HuH-7 cells, suggesting a liver cell-specific defect in replication within this phenotypic group. As indicated in Table 1, five mutant viruses were found to have a *sp* phenotype in both Vero and HuH-7 cells, while 17 viruses had a *sp* phenotype in only HuH-7 cells. Each 5-FU mutant virus clone was compared for a *sp* or *ts* phenotype with three control viruses, 2A-13, wild-type rDEN4, and rDEN4Δ30. The recombinant viruses, rDEN4 and rDEN4Δ30, each had a plaque size in Vero and HuH-7 cells similar to that of DEN4 2A-13, indicating that the Δ30 mutation does not confer a *sp* phenotype (Table 1).

Most of the *sp* 5-FU mutant viruses also had a *ts* phenotype in Vero and/or HuH-7 cells (Table 1) since mutant viruses were initially screened for temperature sensitivity (see Materials and Methods). Temperature sensitivity was defined as a 2.5 or 3.5 $\log_{10}$PFU/ml reduction in virus titer in Vero or HuH-7 cells, respectively, at restrictive temperature compared to the permissive temperature of 35°C as previously defined (Blaney *et al.,* 2001). Three mutant viruses (574, 1269, and 1189) were *sp* and *ts* in both Vero and HuH-7 cells, while nine mutant viruses (506–326 in Table 1) were found to be *ts* in both cell types but *sp* only in HuH-7 cells. Four viruses (1104, 952, 738, and 1083) were found to have a wild-type phenotype in Vero cells but were both *sp* and *ts* in HuH-7 cells. These four mutant viruses each had a 6000- to 600,000-fold reduction in virus titer at 39°C in HuH-7 cells with only a 6- to 40-fold reduction at 39°C in Vero cells. Finally, *sp* mutant viruses were identified which did not have a *ts* phenotype in either cell line; two of these viruses (569 and 761) were *sp* in both Vero and HuH-7 cells and four viruses (1096–1012) were *sp* in only HuH-7 cells (Table 1). These six viruses with only *sp* phenotypes were most likely found in the initial temperature sensitivity screen in a 96-well plate format because of their restricted growth properties at 39°C, which did not permit detection by immunostaining. As described previously, the Δ30 mutation did not confer temperature sensitivity in either cell line (Blaney *et al.,* 2001).

The *sp* 5-FU mutant viruses have restricted replication in suckling mouse brain

The 22 *sp* DEN4 5-FU mutant viruses were evaluated for their ability to replicate in the brain of one-week-old suckling mice. As a marker for *in vivo* attenuation, their level of replication was compared with that of the paral-

TABLE 1

Temperature-Sensitive (*ts*) and Mouse Brain Attenuation (*att*) Phenotypes of 5-FU DEN4 Mutant Viruses Which Also Exhibit a Small Plaque (*sp*) Phenotype

Phenotype					Mean virus titer (log_{10}PFU/ml) at indicated temp. (°C)										Replication in suckling mice[b]		
sp		*ts*			Vero cells					HuH-7 cells							
Vero	HuH-7	Vero	HuH-7	Virus	35	37	38	39	Δ[a]	35	37	38	39	Δ	*n*	Mean virus titer ± SE (log_{10}PFU/g brain)	Mean log_{10} reduction from *wt*[c]
—	—	—	—	2A-13	7.9	7.5	7.7	7.2	0.7	7.9	7.7	7.3	6.9	1.0	66	6.6 ± 0.1[d]	—
—	—	—	—	rDEN4	7.9	7.6	7.7	7.3	0.6	8.1	7.6	7.5	6.7	1.4	66	6.1 ± 0.1[d]	—
—	—	—	—	rDEN4Δ30	7.3	6.6	6.6	6.1	1.2	7.3	7.2	6.9	5.9	1.4	64	5.6 ± 0.1[d]	0.5
+	+	+	+	574	6.6[f]	5.5	3.8	≤1.6[e]	≥5.0	6.6[f]	4.9	5.0	≤1.6	≥5.0	6	2.1 ± 0.1	5.1
+	+	+	+	1269	5.3[f]	4.8	3.9	≤1.6	≥3.7	4.0[f]	2.4	2.0	<1.6	≥2.4	6	2.7 ± 0.2	4.1
+	+	+	+	1189	6.3[f]	5.2	4.5	3.8	2.5	5.5[f]	3.7	2.3	≤1.6	≥3.9	12	3.2 ± 0.4	3.7
+	+	—	—	569	5.8[f]	5.6	5.6	3.7	2.1	6.2[f]	6.0	5.7	5.0	1.2	12	1.9 ± 0.1	4.6
+	+	—	—	761	5.0[f]	4.7	4.2	2.7	2.3	5.6[f]	5.3	4.5	2.6	3.0	12	2.0 ± 0.1	4.2
—	+	+	+	506	7.0	6.8	5.6	2.6	4.4	6.7[f]	4.3	≤1.6	2.0	4.7	6	2.2 ± 0.1	4.7
—	+	+	+	1136	5.1	4.2	2.6	≤1.6	≥3.5	5.7[f]	3.0	3.0	≤1.6	≥4.1	6	2.9 ± 0.3	4.5
—	+	+	+	1029	6.9	5.8	5.8	2.9	4.0	7.0[f]	5.8	5.2	2.5	4.5	6	2.2 ± 0.1	4.2
—	+	+	+	1081	6.9	5.8	4.7	3.9	3.0	5.8[f]	4.1	3.3	1.9	3.9	12	2.6 ± 0.2	3.9
—	+	+	+	529	6.9	6.5	5.9	4.0	2.9	7.1[f]	5.3	4.4	≤1.6	≥5.5	6	3.1 ± 0.7	3.8
—	+	+	+	1114	6.7	6.4	6.2	2.5	4.2	5.7[f]	3.0	2.9	1.9	3.8	6	2.7 ± 0.3	3.7
—	+	+	+	922	7.3	7.2	6.8	3.8	3.5	7.4[f]	5.3	4.1	3.0	4.4	12	3.5 ± 0.1	2.9
—	+	+	+	311	6.9	5.9	4.3	1.5	5.4	7.1[f]	5.4	3.6	≤1.6	≥5.5	12	6.1 ± 0.3	0.9
—	+	+	+	326	6.6	5.7	4.5	3.1	3.5	7.0[f]	5.5	4.1	2.0	5.0	6	6.0 ± 0.1	0.9
—	+	—	+	1104	7.1	6.8	6.8	6.1	1.0	7.2[f]	6.4	5.8	2.8	4.4	6	2.2 ± 0.1	4.7
—	+	—	+	952	7.1	7.0	6.7	5.6	1.5	7.3[f]	6.3	5.6	3.0	4.3	6	2.4 ± 0.3	4.5
—	+	—	+	738	6.5	6.0	5.9	5.7	0.8	6.9[f]	6.1	5.0	3.1	3.8	12	4.4 ± 0.4	2.3
—	+	—	+	1083	7.4	7.3	7.4	5.8	1.6	7.4[f]	6.6	4.5	≤1.6	≥5.8	12	4.5 ± 0.4	2.0
—	+	—	—	1096	7.5	7.1	6.9	5.5	2.0	7.5[f]	6.6	5.6	4.8	2.7	6	2.9 ± 0.2	3.5
—	+	—	—	1021	7.0	6.9	6.6	6.3	0.7	6.9[f]	5.7	4.4	4.0	2.9	6	3.9 ± 0.6	2.6
—	+	—	—	1023	6.6	6.4	6.0	5.8	0.8	6.1[f]	5.6	4.7	3.3	2.8	12	4.2 ± 0.3	2.3
—	+	—	—	1012	7.5	7.1	7.0	5.7	1.8	7.4[f]	6.8	6.8	5.6	1.8	6	6.1 ± 0.1	0.8

[a] Reduction in mean virus titer (log_{10}PFU/ml) at 39°C compared to permissive temperature (35°C).

[b] Groups of six suckling mice were inoculated i.c. with 10^4 PFU virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[c] Determined by comparing mean viral titers of mice inoculated with mutant virus and concurrent 2A-13 wild type (*wt*) virus control ($n = 6$ or 12).

[d] Average of 11 experiments with a total of 64 to 66 mice per group.

[e] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature (35°C).

[f] Small plaque size at 35°C; small plaques have a diameter of <1.0 mm compared to wild-type plaque diameter of 1.5–2.0 mm in Vero cells, or a diameter of <0.4 mm compared to wild-type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

lel-passaged control virus with a wild-type phenotype, 2A-13 (Table 1). Nineteen of 22 *sp* mutant viruses had a greater than 100-fold reduction in virus replication in the brain of suckling mice compared to 2A-13 and nine viruses had a reduction of greater than 10,000-fold.

The five mutant viruses which were *sp* in both Vero and HuH-7 cells were 5000- to 100,000-fold restricted in replication compared to 2A-13. Two of these mutant viruses, 569 and 761, were not *ts* in either cell line but had a reduction in virus titer of greater than 10,000-fold in mouse brain, suggesting that the *sp* phenotype in both Vero and HuH-7 cells may be an important surrogate marker for attenuated replication in suckling mouse brain. 5-FU mutant viruses which were *sp* in only HuH-7 cells had a more variable range of replication in mouse brain. Three viruses had a mean reduction in virus titer of less than 10-fold when compared to 2A-13 virus. However, 8 of 13 viruses which were *ts* in Vero and/or HuH-7 cells but *sp* in only HuH-7 cells had a greater than 5000-fold reduction in virus replication. The results of the *in vivo* replication analysis of the previously described 20 *ts* 5-FU mutant viruses (Blaney *et al.,* 2001) and the 22 *sp* mutant viruses are summarized in Table 2. Mutant viruses with both a *sp* and *ts* phenotype were found to have a significantly greater level of attenuation in the brain of suckling mice when compared to viruses with only a *ts* phenotype.

Sequence analysis of the *sp* 5-FU mutant viruses

To initiate an analysis of the genetic basis of the *ts*, *sp*, or *att* phenotype of the 22 *sp* mutant viruses, the com-

TABLE 2

Viruses with both *ts* and *sp* Phenotypes are More Restricted in Replication in Mouse Brain Than Those with Only a *ts* Phenotype

Cell culture phenotype	Number of viruses	Mean $\log_{10}$ reduction in virus titer from control[a,b]
ts[c]	20	2.1 ± 0.2
sp	6	3.0 ± 0.6
ts/sp	16	3.5 ± 0.3

[a] Determined by comparing mean viral titers of groups of mice inoculated with mutant virus and concurrent 2A-13 parallel-passaged control virus.

[b] Significant difference between *ts* group and *ts/sp* group, Tukey–Kramer test ($P < 0.05$).

[c] 20 *ts* mutant viruses without an *sp* phenotype were previously described (Blaney *et al.,* 2001).

plete nucleotide sequence of each virus genome was determined and is summarized in Table 3 (*sp* in Vero and HuH-7 cells) and Table 4 (*sp* in only HuH-7 cells). All identified mutations were nucleotide substitutions, as deletions or insertions were not observed. Point mutations were distributed throughout the genome, including the 3′-UTR as well as in all coding regions. Because all 5-FU mutant viruses were found to have at least two mutations (two to six), the observed phenotypes cannot be directly attributed to a specific mutation. The majority of *sp* viruses also contained translationally silent point mutations (none to four) in the structural or nonstructural coding regions. However, these silent mutations are not expected to contribute to the observed phenotypes. Six of the 22 *sp* mutant viruses (Tables 3 and 4) were found to have mutations in only the NS genes and/or the 3′-UTR, indicating that the *sp* phenotype can be conferred by mutations outside of the structural genes.

Presence of identical mutations in multiple 5-FU mutant viruses

Analysis of the complete nucleotide sequence data for the 5-FU mutant viruses identified several repeated mutations which were present in two or more viruses. Such mutations were also identified previously during our analysis of twenty 5-FU mutant viruses with a *ts* but not *sp* phenotype (Blaney *et al.,* 2001). Because these mutations occurred in viruses together with additional mutations, the contribution of the repeated mutations to the observed *sp*, *ts*, and *att* phenotypes remains unclear. Table 5 lists the repeated mutations found among the 20

TABLE 3

Nucleotide and Amino Acid Differences of the 5-FU Mutant DEN4 Viruses Which Produce Small Plaques in Both Vero and HuH-7 Cells

	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
Virus	Nucleotide position	Gene/region	Nucleotide change	Amino acid change[a]	Nucleotide position	Gene	Nucleotide change
569	826	prM	G > A	R242K	1946	E	C > U
	832	prM	C > U	P244L			
	7546	NS4B	C > U	A2482V			
	10,275	3′ UTR	A > U	n/a			
	10,279	3′ UTR	A > U	n/a			
574	1455	E	G > U	V452F	1349	E	C > U
	1963	E	U > C	V621A			
	3880	NS2A	A > G	K1260R			
	7546	NS4B	C > U	A2482V			
	7615	NS5	A > G	N2505S			
	10,413	3′ UTR	A > G	n/a			
761	424	C	U > C	I108T	None		
	2280	E	U > C	F727L			
	7131	NS4B	A > G	T2344A			
	7486	NS4B	A > G	N2462S			
1189[b]	3303	NS1	A > G	R1068G	6719	NS4A	U > C
	4812	NS3	G > A	V1571I			
	5097	NS3	G > A	D1666N			
	7182	NS4B	G > A	G2361S			
1269	2112	E	U > C	F671L	542	prM	C > U
	3256	NS1	G > A	G1052E			
	3993	NS2A	U > C	F1298L			
	7183	NS4B	G > U	G2361V			

[a] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102–104). Wild-type amino acid on left of amino acid position; mutant amino acid on right.

[b] Virus contains missense mutations in only the nonstructural genes.

TABLE 4

Nucleotide and Amino Acid Differences of the 5-FU Mutant DEN4 Viruses Which Produce Small Plaques in Only HuH-7 Cells

	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
Virus	Nucleotide position	Gene/region	Nucleotide change	Amino acid change[a]	Nucleotide position	Gene	Nucleotide change
311	1519	E	A > G	N473S	6761	NS4A	C > U
	2305	E	G > A	R735K	10,070	NS5	U > C
	4896	NS3	G > U	A1599S			
326	1587	E	C > U	P496S	1523	E	G > A
	7546	NS4B	C > U	A2482V	6080	NS3	U > C
					10,070	NS5	U > C
506	1455	E	G > U	V452F	3887	NS2A	A > G
	1902	E	G > A	V601M	5789	NS3	G > C
	7546	NS4B	C > U	A2482V			
	10,275	3′ UTR	A > U	n/a			
529	777	prM	U > C	S226P	None		
	4641	NS3	A > G	I1514V			
	7153	NS4B	U > C	V2351A			
	8245	NS5	U > C	I2715T			
	10,279	3′ UTR	A > C	n/a			
738[b]	3540	NS2A	G > A	E1147K	None		
	7162	NS4B	U > C	L2354S			
922[b]	4306	NS2B	A > G	N1402S	7736	NS5	G > A
	5872	NS3	C > U	T1924I			
	7163	NS4B	A > U	L2354F			
	10,279	3′ UTR	A > C	n/a			
952	1449	E	G > U	V450L	None		
	1455	E	G > U	V452F			
	7546	NS4B	C > U	A2482V			
	7957	NS5	U > C	V2619A			
	9543	NS5	A > G	I3148V			
1012	1542	E	A > G	K481E	953	E	A > G
	7162	NS4B	U > C	L2354S	1205	E	G > A
	10,542	3′ UTR	A > G	n/a	4425	NS2B	U > C
1021	2314	E	U > C	I738T	665	prM	C > A
	3205	NS1	C > U	A1035V	5750	NS3	C > U
	4029	NS2A	U > C	C1310R	9959	NS5	C > U
	7163	NS4B	A > C	L2354F			
	10,275	3′ UTR	A > U	n/a			
	10,279	3′ UTR	A > U	n/a			
1023	2283	E	G > A	G728R	1001	E	C > U
	7182	NS4B	G > A	G2361S	1958	E	A > G
					3873	NS2A	U > C
					8486	NS5	C > U
1029	850	prM	C > U	A250V	3867	NS2A	C > U
	3087	NS1	A > G	T996A			
	4891	NS3	U > C	I1597T			
1081[b]	2650	NS1	A > G	N850S	6326	NS3	C > U
	7163	NS4B	A > U	L2354F	9146	NS5	C > U
1083[b]	3702	NS2A	G > A	A1201T	3353	NS1	A > G
	7153	NS4B	U > C	V2351A	6155	NS3	G > A
	10,634	3′ UTR	U > C	n/a			
1096	892	prM	G > A	R264Q	665	prM	C > A
	7163	NS4B	A > C	L2354F	4427	NS2B	G > A
	8659	NS5	C > U	P2853L			
1104	1692	E	G > A	V531M	None		
	5779	NS3	C > U	A1893V			
	7546	NS4B	C > U	A2482V			

TABLE 4—*Continued*

Virus	Mutations in UTR or in coding regions that result in an amino acid substitution				Mutations in coding regions that do not result in an amino acid substitution		
	Nucleotide position	Gene/region	Nucleotide change	Amino acid change[a]	Nucleotide position	Gene	Nucleotide change
1114	709	prM	A > G	K203R	1076	E	U > C
	3693	NS2A	A > G	I1198V	1182	E	C > U
	4614	NS3	U > C	F1505L	5690	NS3	C > U
	7546	NS4B	C > U	A2482V			
	9942	NS5	A > G	T3281A			
1136[b]	3771	NS2A	A > G	R1224G	5621	NS3	A > G
	4891	NS3	U > C	I1597T			
	10,275	3′ UTR	A > U	n/a			

[a] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102–104). Wild-type amino acid on left of amino acid position; mutant amino acid on right.

[b] Viruses that contain missense mutations in only the nonstructural genes and/or mutations in the UTRs.

ts (not *sp*) mutant viruses described previously (Blaney *et al.,* 2001) and the 22 *sp* mutant viruses described here. Repeated mutations were identified in the following genes: two in E, two in NS3, five in NS4B, one in NS5, and two in the 3′-UTR. Interestingly, within a 30 nucleotide region of NS4B (nt 7153–7182), there were five different nucleotide substitutions which were found in 16 viruses. Also at nt 7546 in NS4B, an amino acid substitution (Ala → Val) was found in 10 different 5-FU mutant viruses. The significance of these repeated mutations in NS4B as well as in other DEN4 genomic regions remains unclear, but a reasonable explanation for this phenomenon is that these mutations are involved in adaptation of DEN4 virus for efficient growth in Vero cells. Alternatively, these repeated mutations could represent loci of genetic instability which have no correlation with adaptation to growth in Vero cells.

TABLE 5

Putative Vero Cell Adaptation Mutations Derived from the Set of 5-FU Mutant Viruses

Nucleotide position	Gene/region (a.a. #)[a]	5-FU mutant viruses		
		Nucleotide change	Amino acid change	No. of viruses with the mutation
1455	E (452)	G > U	Val > Phe	5
2280	E (727)	U > C	Phe > Leu	2
4891	NS3 (1597)	U > C	Ile > Thr	2
4995	NS3 (1599)	U > C	Ser > Pro	8
7153	NS4B (2351)	U > C	Val > Ala	3
7162	NS4B (2354)	U > C	Leu > Ser	4
7163	NS4B (2354)	A > U or C	Leu > Phe	7
7182	NS4B (2361)	G > A	Gly > Ser	2
7546	NS4B (2482)	C > U	Ala > Val	10
7630	NS5 (2510)	A > G	Lys > Arg	1
10,275	3′ UTR	A > U	n/a[b]	6
10,279	3′ UTR	A > C	n/a	4

[a] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102–104) as residue 1.

[b] Not applicable.

Genetic basis of *ts*, *sp*, and *att* phenotypes in suckling mice for selected 5-FU mutant viruses

As described above, six 5-FU mutant viruses (Tables 3 and 4) were found to have coding mutations in only the NS genes and/or nucleotide substitutions in the 3′-UTR: 5-FU mutant 738, 922, 1081, 1083, 1136, and 1189. Because of our particular interest in *att* mutations outside of the structural gene region, we sought to identify the individual mutations among these viruses which confer the observed phenotypes. Seventeen mutations identified by sequence analysis resulted in a coding change or a nucleotide change in the UTR and each was engineered into an individual DEN4 cDNA clone. Virus was successfully recovered and propagated containing each defined mutation and tested for efficiency of plaque formation in Vero and HuH-7 cells at various temperatures, plaque-size phenotype, and growth properties in suckling mice as was performed for the parental 5-FU mutant viruses.

Table 6 lists the phenotypes of the six 5-FU mutant parent viruses followed by those of the 17 rDEN4 viruses encoding single mutations present in the parent virus. For example, 5-FU mutant 1189 (parent), which was *ts* and *sp* in both cell lines and had an almost 10,000-fold reduction in replication in suckling mouse brain, contained four coding mutations at nt position 3303 in NS1, 4812 and 5097 in NS3, and 7182 in NS4B. Analysis of the four rDEN4 viruses containing each of these mutations indicated that rDEN4-5097 had a *ts*, *sp*, and *att* phenotype, while rDEN4-3303, rDEN4-4812, and rDEN4-7182 had no discernible phenotypes, indicating that the mutation at nt 5097 was responsible for the phenotype

TABLE 6

sp, ts, and Mouse Attenuation Phenotypes of rDEN4 Mutant Viruses Encoding Single Mutations Identified in Six *sp* 5-FU Mutant Viruses

5-FU mutant virus	Virus	Gene/region containing mutation	Mean virus titer (log_{10}PFU/ml) at indicated temp (°C)						Replication in suckling mice[b]		Replication in HuH-7-SCID mice[d]		
			Vero cells			HuH-7 cells							
			35	39	Δ[a]	35	39	Δ	*n*	Mean virus titer ± SE (log_{10}PFU/g brain)	Mean log_{10}-unit reduction from value for wt[c]	*n*	Mean peak virus titer ± SE (log_{10}PFU/ml serum)	Mean log_{10}-unit reduction from value for wt[c]
	2A-13		7.6	7.1	0.5	7.8	6.6	1.2	30	6.5 ± 0.1	—	29	6.8 ± 0.2	—
	rDEN4		7.6	6.8	0.8	8.0	6.7	1.3	54	5.8 ± 0.1	—	32	6.3 ± 0.2	—
	rDEN4Δ30		7.6	6.9	0.7	7.7	5.6	2.1	30	5.6 ± 0.1	0.2	18	5.4 ± 0.2	0.9
738	Parent		6.5	5.7	0.8	6.9[f]	3.1[e]	3.8	12	4.4 ± 0.4	**2.3**	9	5.4 ± 0.7	1.9
	rDEN4-3540	NS2A	6.9	5.1	1.8	7.4	3.7	3.7	12	4.1 ± 0.3	**1.7**	5	6.1 ± 0.3	(+)0.1
	rDEN4-7162	NS4B	7.2	6.8	0.4	7.4	6.6	0.8	8	5.6 ± 0.3	0.3	5	6.8 ± 0.6	0.3
922	Parent		7.3	3.8	3.5	7.4[f]	3.0	4.4	12	3.5 ± 0.1	**2.9**	6	6.2 ± 0.2	0.4
	rDEN4-4306	NS2B	5.0[f]	2.2	2.8	5.6[f]	≤1.6	>4.0	12	1.7 ± 0.1	**4.1**	5	5.2 ± 0.6	1.1
	rDEN4-5872	NS3	5.7	2.5	3.2	6.5[f]	≤1.6	>4.9	12	4.5 ± 0.3	1.3	5	6.2 ± 0.5	0.1
	rDEN4-7163	NS4B	7.8	7.2	0.6	8.0	7.4	0.6	6	6.2 ± 0.2	(+)0.1	6	5.8 ± 0.6	(+)0.2
	rDEN4-10279	3′ UTR	6.9	5.7	1.2	7.7	5.7	2.0	6	4.8 ± 0.2	0.7	4	6.7 ± 0.2	0.4
1081	Parent		6.9	3.9	3.0	5.8[f]	1.9	3.9	12	2.6 ± 0.2	**3.9**	4	4.2 ± 0.5	**2.4**
	rDEN4-2650	NS1	5.1	3.0	2.1	5.5[f]	2.8	2.7	12	3.0 ± 0.3	**2.8**	6	4.7 ± 0.5	**2.2**
	rDEN4-7163	NS4B	7.8	7.2	0.6	8.0	7.4	0.6	6	6.2 ± 0.2	(+)0.1	6	5.8 ± 0.6	(+)0.2
1083	Parent		7.4	5.8	1.6	7.4[f]	≤1.6	≥5.8	12	4.5 ± 0.4	**2.0**	9	4.4 ± 0.3	**2.9**
	rDEN4-3702	NS2A	6.8	5.6	1.2	7.6	4.7	2.9	18	4.9 ± 0.3	0.9	7	6.3 ± 0.3	0.2
	rDEN4-7153	NS4B	7.7	7.2	0.5	8.0	6.9	1.1	6	5.7 ± 0.1	0.2	4	5.9 ± 0.7	0.1
	rDEN4-10634	3′ UTR	4.9	1.6	3.3	5.7[f]	≤1.6	≥4.1	12	2.4 ± 0.3	**3.4**	7	3.3 ± 0.4	**3.6**
1136	Parent		5.1	≤1.6	≥3.5	5.7[f]	≤1.6	≥4.1	6	2.9 ± 0.3	**4.5**	7	4.5 ± 0.4	1.2
	rDEN4-3771	NS2A	7.0	4.6	2.4	7.6[f]	3.7	3.9	12	2.6 ± 0.4	**3.2**	4	6.4 ± 0.2	(+)0.1
	rDEN4-4891	NS3	7.1	≤1.6	>5.5	7.4[f]	≤1.6	>5.8	12	2.5 ± 0.3	**3.5**	6	6.0 ± 0.5	0.3
	rDEN4-10275	3′ UTR	6.9	5.8	1.1	7.1	5.2	1.9	6	5.0 ± 0.3	0.5	4	6.7 ± 0.3	0.4
1189	Parent		6.3[f]	3.8	2.5	5.5[f]	≤1.6	≥3.9	12	3.2 ± 0.4	**3.7**	13	2.3 ± 0.3	**3.8**
	rDEN4-3303	NS1	6.1	4.8	1.3	6.6	3.9	2.7	8	5.7 ± 0.4	0.2	4	6.3 ± 0.3	0.8
	rDEN4-4812	NS3	7.0	6.3	0.7	7.1	6.3	0.8	12	4.8 ± 0.2	1.0	5	6.1 ± 0.5	(+)0.5
	rDEN4-5097	NS3	5.0[f]	≤1.6	>3.4	4.6[f]	≤1.6	>3.0	12	1.8 ± 0.1	**4.0**	8	1.9 ± 0.1	**4.3**
	rDEN4-7182	NS4B	7.7	6.9	0.8	7.8	6.8	1.0	6	6.2 ± 0.1	(+)0.1	6	6.3 ± 0.3	(+)0.7

[a] Reduction in mean virus titer (log_{10}PFU/ml) at 39°C compared to permissive temperature (35°C).

[b] Groups of six suckling mice were inoculated i.c. with 10^4 PFU of virus. Brains were removed 5 days later, homogenized, and titered in Vero cells.

[c] Comparison of mean virus titers of mice inoculated with mutant virus and concurrent DEN4 control. Bold denotes ≥50- or ≥100-fold decrease in replication in suckling or SCID-HuH-7 mice, respectively.

[d] Groups of HuH-7-SCID mice were inoculated directly into the tumor with 10^4 PFU virus. Serum was collected on day 6 and 7 and titered in Vero cells.

[e] Underlined values indicate a 2.5 or 3.5 log_{10}PFU/ml reduction in titer in Vero cells or HuH-7 cells, respectively, at indicated temperature when compared to permissive temperature (35°C).

[f] Small plaque size at 35°C; small plaques have a diameter of <1.0 mm compared to wild-type plaque diameter of 1.5–2.0 mm in Vero cells, or a diameter of <0.4 mm compared to wild-type plaque diameter of 0.75 to 1.0 mm in HuH-7 cells.

observed in the 5-FU parent, 1189. The *att* phenotypes of 5-FU mutant parent viruses, 738, 922, 1081, and 1083, were similarly attributed to single mutations; NS2A 3540, NS2B 4306, NS1 2650, and 3′-UTR 10634, respectively. However, two separate mutations (NS2A 3771 and NS3 4891) contributed to the phenotypes of 5-FU mutant virus 1136.

Replication of DEN4 viruses in SCID mice transplanted with HuH-7 cells

To further examine the *in vivo* growth properties of the six 5-FU mutant viruses with mutations in only the NS genes and/or the 3′-UTR and the corresponding 17 rDEN4 mutant viruses, replication was assessed in SCID mice transplanted with HuH-7 cells (SCID-HuH-7). Preliminary experiments indicated that SCID-HuH-7 mice inoculated with DEN4 2A-13 directly into the tumor developed viremia with maximum levels (up to 8.0 log_{10}PFU/ml serum) achieved on Day 6 or 7. Virus could also be detected in brain, liver, and tumor homogenates (data not shown). The level of viremia in SCID-HuH-7 mice infected with parental 5-FU or rDEN4 mutant viruses was compared with that of the parallel-passaged control virus, 2A-13, or rDEN4, respectively. Results of

TABLE 7

Conservation of the *att* Mutations Identified in rDEN4 Viruses within the DEN1, DEN2, and DEN3 Virus Serotypes

			Mouse *att* phenotypes[b]		Identity in DEN serotype[c]		
Mutation (nt number)	Amino acid change[a]	Gene/region	Suckling brain	SCID-HuH-7 serum	1 (WP)	2 (NGC)	3 (H87)
2650	N850S	NS1	√	√	+	+	+
3540	E1147K	NS2A	√	−	+	+	+
3771	R1224G	NS2A	√	−	+	−	+
4306	N1402S	NS2B	√	−	−	−	−
4891	I1597T	NS3	√	−	−	+	+
5097	D1666N	NS3	√	√	+	+	+
10634	n/a	3′ UTR	√	√	+	+	+

[a] Amino acid position in DEN4 polyprotein beginning with the methionine residue of the C protein (nt 102–104) as residue 1. Wild-type amino acid on left of amino acid position; mutant amino acid on right.

[b] *att* replication in suckling mouse brain defined as ≥50-fold decrease in virus titer in brain on day 5 postinfection when compared to wild-type rDEN4. *att* replication in SCID-HuH-7 mice defined as a ≥100-fold decrease in peak serum virus titer on day 6 and 7 postinfection when compared to wild-type rDEN4.

[c] Plus indicates presence of exact DEN4 wild-type amino acid or nucleotide (UTR) in other serotypes. Minus indicates the amino acid or nt of DEN4 wild-type is not identical in the other serotypes. Strains: DEN1 Western Pacific, DEN2 New Guinea C, DEN3 H87.

four separate experiments indicated that the vaccine candidate, rDEN4Δ30, had an almost 10-fold reduction in virus replication compared to wild-type rDEN4 (Table 6). Three 5-FU mutant viruses had a greater than 100-fold reduction in viremia in the SCID-HuH-7 mice compared to wild-type 2A-13 virus: 1081, 1083, and 1189. Analysis of the genetic basis of the *att* phenotype in these parent 5-FU mutant viruses identified three individual mutations in NS1, NS3, and the 3′-UTR which conferred at least a 100-fold reduction in viremia. Specifically, rDEN4-2650 (NS1), rDEN4-5097 (NS3), and rDEN4-10634 (3′-UTR) manifested a 2.2, 3.6, and 4.3 $\log_{10}$PFU/ml reduction in peak titer of viremia compared to rDEN4, respectively. These mutations also conferred the *att* phenotype in suckling mouse brain.

Conservation of *att* mutations in DEN1, -2, and -3

Based on the moderate genetic homology of the four DEN virus serotypes, it was of interest to determine whether the sites of the *att* mutations identified in DEN4 are conserved in DEN1, -2, and/or -3. The phenotypes and amino acid or UTR nucleotide conservation at the seven specific mutant loci which conferred an *att* phenotype in suckling mouse brain or SCID-HuH-7 mice are summarized in Table 7. The wild-type DEN4 virus sequence at six of the seven mutant loci was at a conserved site in at least two DEN virus serotypes, and four were conserved in all serotypes. Each of the mutations that restricted replication in the SCID-HuH-7 mice (2650 in NS1, 5097 in NS3, 10634 in the 3′-UTR) was at a conserved site in each of the four DEN virus serotypes.

DISCUSSION

As part of a molecular genetic vaccine strategy, we sought to develop attenuating mutations that might be useful in the development of a live-attenuated tetravalent dengue virus vaccine. Specifically, mutations which restrict replication of the vaccine virus in human liver cells were generated since there was some residual virulence of the rDEN4Δ30 vaccine candidate for the liver of humans. Mutant viruses with a *sp* phenotype were sought in both Vero cells and HuH-7 human liver cells to identify host-range mutant viruses that were specifically restricted in replication in HuH-7 cells (*sp* in HuH-7 but not in Vero). Such mutations might be particularly useful in limiting replication of a candidate vaccine in the liver of vaccinees while preserving both efficient replication in Vero cells and immunogenicity *in vivo*.

Several observations from the present study indicate that *sp* mutations confer an *att* phenotype *in vivo*. This is not surprising since attenuation in suckling mouse brain has been reported for live DEN virus vaccine candidates possessing *sp* phenotypes, including the DEN2 PDK-53 and DEN2 PR-159/S-1 vaccine strains (Bhamarapravati and Yoksan, 1997; Butrapet *et al.,* 2000; Eckels *et al.,* 1980; Innis *et al.,* 1988). Each of 22 DEN4 5-FU mutant viruses with a *sp* phenotype (some of which were also *ts*) in either Vero or HuH-7 cells manifested restricted replication in the brains of mice. Six 5-FU mutant viruses with a *sp* phenotype in the absence of a *ts* phenotype were more attenuated in the brains of suckling mice than mutant viruses with solely a *ts* phenotype, suggesting that the *sp* phenotype specifies a greater level of attenuation for mouse brain than does the *ts* phenotype. Sixteen mutant viruses with both a *ts* and a *sp* phenotype had an even greater reduction in replication (Tables 1 and 2), further suggesting that the attenuation conferred by the *ts* and *sp* phenotypes can be additive. Importantly, 17 of the 22 *sp* mutant viruses were host-range *sp* mutant viruses, being *sp* only in HuH-7 cells. Since such muta-

tions could potentially be useful in restricting the replication of a DEN4 virus in human liver cells, we used nucleotide sequence analysis to determine the genetic basis of the *sp* phenotype.

Analysis of the complete genomic sequence of the 22 *sp* DEN4 viruses revealed substitutions in the 3′-UTR as well as coding mutations in all genes except NS4A. It was first noted that several specific mutations were present in two or more of the 22 *sp* DEN4 mutant viruses and that many of these same mutations were also previously identified among the set of 20 *ts* DEN4 mutant viruses (Blaney *et al.,* 2001). Since flaviviruses can rapidly accumulate mutations during passage in tissue culture (Dunster *et al.,* 1999; Mandl *et al.,* 2001), many of these overrepresented mutations, previously referred to as putative Vero cell adaptation mutations (Blaney *et al.,* 2001), likely promote efficient replication in Vero cells and were selected unintentionally during the biological cloning of the mutant viruses. The effect of these mutations on DEN virus replication in Vero cells, the proposed substrate for vaccine manufacture, is currently being evaluated.

It was next noted that six of the 22 *sp* DEN4 virus mutant viruses contained coding mutations in only the NS genes and/or nucleotide substitutions in the 3′-UTR, and that five of these six viruses had a host-range *sp* phenotype. Such mutations are particularly well suited for construction of chimeric viruses consisting of an attenuated DEN4 genetic background bearing the wild-type structural genes of another DEN virus serotype (Bray and Lai, 1991; Durbin *et al.,* 2001; Huang *et al.,* 2000) or of another flavivirus such as West Nile virus (WNV) or Langat virus (LAN) (Pletnev and Men, 1998; Pletnev *et al.,* 2002). For this reason we focused our attention on determining the genetic basis of the *sp* phenotype of these six viruses which contained a total of 17 different mutations. rDEN4 viruses were engineered that contained each of these mutations as separate mutations, and the cell culture and *in vivo* phenotypes of the 17 rDEN4 viruses were then determined. Seven of these mutations were identified as attenuating mutations that conferred restricted replication in suckling mouse brain ranging from 50-fold to 10,000-fold below that observed in mice infected with wild-type rDEN4.

We next sought to determine whether any of the six 5-FU mutant viruses bearing mutations in only the NS genes and UTR or if any of their 17 rDEN4 single-mutation derivatives were restricted in replication in the liver of an infected animal. Since DEN viruses replicate poorly in the liver of mice and corresponding studies are impractical to conduct in nonhuman primates, an animal model that evaluates the *in vivo* level of replication of DEN virus in liver cells was developed based on a recent report examining the replication of DEN virus in SCID mice transplanted with a continuous cell line of human liver tumor cells (An *et al.,* 1999). SCID mice transplanted with human continuous cell lines, primary cells, or organized tissues have similarly been used to study the replication of other viruses which lack a suitable small animal model (Mosier, 2000). In our study, SCID mice were transplanted with HuH-7 cells since DEN4 virus replicated efficiently in these cells in tissue culture and since these were the cells used to define the host-range phenotype. Although each of the six 5-FU mutant viruses evaluated in SCID-HuH-7 mice had an *sp* phenotype in HuH-7 cells, only three viruses manifested reduced replication in these mice. This observation indicated that analysis of virus replication in SCID-HuH-7 mice served as a more stringent test to define the *att* phenotype in these liver cells compared to the *sp* phenotype in cell culture.

An analysis of the genetic basis of the phenotypes specified by the mutations in the 5-FU mutant viruses that manifested restricted replication in SCID-HuH-7 mice indicated that (1) three separate mutations conferred the *att* phenotype; (2) these mutations were located in two proteins, NS1 and NS3, and in the 3′-UTR; (3) these three mutations were fully responsible for each of the cell culture (*ts* or *sp*) and *in vivo* (attenuation in mouse brain and SCID-HuH-7 mice) phenotypes of the parent viruses; and (4) two of the three mutations (2650 and 10634) specify the host-range *sp* phenotype (*sp* on HuH-7 only) and might therefore be particularly useful in a vaccine virus. Although the relevance of such SCID-transplant models to virus replication and disease in humans is unknown, the identification of three novel mutations which restrict DEN4 virus replication in SCID-HuH-7 mice will allow us to examine the correlation between the *att* phenotype in SCID-HuH-7 mice with that in rhesus monkeys or humans. Such mutations, specifically the host-range *sp* mutations, might be useful in conjunction with the Δ30 mutation to decrease the residual virulence of rDEN4Δ30 for the human liver, and studies are in progress to construct such rDEN4 viruses and evaluate them in monkeys and humans.

The present studies have not elucidated the mechanisms by which the three *sp* mutations restrict DEN4 replication in cell culture and suckling or SCID-HuH-7 mice, although previous studies of NS1, NS3, and the 3′-UTR may yield potential insights (Rice, 1996). Previously, we described a *ts*-attenuating mutation at nucleotide 4995 in NS3, resulting in a substitution at amino acid 158 located in the N-terminal half of the protein which contains the protease domain (Blaney *et al.,* 2001). However, the *sp* mutation 5097 described in this study, which results in an amino acid substitution (Asp to Asn) at position 192 of NS3, is localized in conserved motif I (amino acid 188–205) of the C-terminal region containing the nucleoside triphosphatase (NTPase) and RNA helicase domains (Gorbalenya *et al.,* 1990; Kadare and Haenni, 1997; Kim *et al.,* 1998; Li *et al.,* 1999). This motif, involved in binding the β and γ phosphate groups of NTP,

is highly conserved in flaviviruses and the Asp at amino acid position 192 is conserved in DEN1–4, YF, WNV, LAN, Kunjin virus, Japanese encephalitis virus, tick-borne encephalitis virus, and Powasson virus (Chang, 1997). Two substitutions in motif I have previously been shown to abolish ATPase and RNA helicase activities (Matusan *et al.,* 2001), suggesting that the 5097 mutation may behave similarly. It is possible that the two mutations in NS3, namely, the 4995 and 5097 mutations which are present in distinct functional domains of NS3, could be combined using reverse genetics to yield a new rDEN4 virus, perhaps with a synergistic effect on the *att* phenotype.

The mutation at nucleotide 2650 results in an amino acid substitution (Asn to Ser) at position 76 of NS1, a 352 amino acid glycosylated dimer which localizes to intracellular membranes, the cell surface, and is also present in a secreted form (Brandt *et al.,* 1970; Mackenzie *et al.,* 1996; Muylaert *et al.,* 1997; Rice, 1996). The mechanism of the phenotype specified by this mutation cannot be inferred at this time. Interestingly, the final *sp* mutation which conferred an *att* phenotype in SCID-HuH-7 mice results in a noncoding change in the 3′-UTR at nucleotide 10,634 (U to C). Substitution or deletion *att* mutations have been found in the noncoding regions of flaviviruses (Butrapet *et al.,* 2000; Cahour *et al.,* 1995; Mandl *et al.,* 1998; Men *et al.,* 1996; Pletnev, 2001), as well as the Sabin poliovirus vaccine strains (Macadam *et al.,* 1994) and respiratory syncytial virus (Whitehead *et al.,* 1998). The 10,634 mutation is located 15 nt from the 3′-terminus of the genome and is not paired to a complementary nucleotide in the predicted stem-loop secondary structure (Proutski *et al.,* 1997; Shurtleff *et al.,* 2001). Mutations in the 3′-UTR could possibly restrict replication by multiple mechanisms including alterations in the secondary structure which could abrogate binding of viral or cellular factors required for efficient RNA synthesis or interference with RNA packaging in virion assembly.

The *sp* mutations identified among the 5-FU mutant viruses could potentially be used in several different approaches for the development of DEN virus vaccine strains. As described above for the generation of antigenic chimeric viruses, one or more *sp* attenuating mutations could be evaluated in the context of the DEN4Δ30 genetic background as a means to augment the *att* phenotype in humans already conferred by the Δ30 mutation. A second approach would be to introduce a *sp*-attenuating mutation, with or without Δ30, into infectious cDNA clones of the other three DEN serotypes. The ability to transfer mutations among genetically related viruses and maintain similar *att* phenotypes has been previously demonstrated (Skiadopoulos *et al.,* 1999). Interestingly, six of seven mutations with *att* phenotypes in suckling mouse brain were found to be conserved in at least two DEN virus serotypes (Table 7), suggesting that one or more of these mutations could be used to attenuate DEN1, -2, and -3 virus. Importantly, the amino acid and nucleotide sequence of each of the mutations in DEN4 NS1, NS3, and the 3′-UTR with an *att* phenotype in SCID-HuH-7 mice are conserved among the other three DEN virus serotypes. These distinct strategies could potentially be used as separate or complementary approaches to the construction of a tetravalent DEN virus vaccine, underlining the importance of the identification of a large panel of *att* mutations within the DEN viruses.

MATERIALS AND METHODS

Cells and viruses

WHO Vero cells (African green monkey kidney cells) were grown in MEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.05 mg/ml gentamicin (Invitrogen). HuH-7 cells (human hepatoma cells) (Nakabayashi *et al.,* 1982) were maintained in D-MEM/F-12 (Invitrogen) supplemented with 10% FBS, 1 mM L-glutamine, and 0.05 mg/ml gentamicin.

DEN4 2A virus is a wild-type virus derived from a cDNA clone of DEN4 strain 814669 (Dominica, 1981) (Lai *et al.,* 1991; Mackow *et al.,* 1987). The nucleotide sequence of DEN4 2A, the parent of the 5-FU mutant viruses, was previously assigned GenBank Accession No. AF375822 (Blaney *et al.,* 2001). The DEN4 vaccine candidate, 2AΔ30 (Durbin *et al.,* 2001), contains a 30-nt deletion in the 3′-UTR which removes nucleotides 10,478–10,507 (Men *et al.,* 1996). The cDNA clones p4, a modified derivative of the DEN4 2A cDNA clone, and p4Δ30 were used to generate recombinant wild-type and attenuated viruses, rDEN4 and rDEN4Δ30, respectively (Durbin *et al.,* 2001). GenBank accession numbers were previously assigned as follows (virus: Accession No.): DEN4 strain 814669: AF326573; 2AΔ30: AF326826; rDEN4: AF326825; rDEN4Δ30: AF326827.

Generation and biological cloning of mutant viruses with a *sp* phenotype

The generation of 1248 virus clones from a pool of 5-fluorouracil-mutagenized DEN4 2A has been previously described (Blaney *et al.,* 2001). Briefly, monolayers of Vero cells were infected with DEN4 2A at a multiplicity of infection (m.o.i.) of 0.01 and overlaid with MEM supplemented with 2% FBS and 1 mM 5-FU (Sigma, St. Louis, MO), which reduced replication of DEN4 2A 100-fold. Vero cells in 96-well plates were inoculated with the 5-FU-treated virus suspension, and virus clones were harvested from plates receiving terminally diluted virus. A total of 1248 virus clones were generated from the cultures treated with 1 mM 5-FU. Two virus clones, 2A-1 and 2A-13, were generated in the same manner from control cultures not treated with 5-FU and served as parallel-passaged control viruses with a wild-type phenotype.

Evaluation of *in vitro* plaque size and temperature sensitivity

The 1248 5-FU-mutagenized virus clones were screened for temperature sensitivity by assessing virus replication at 35°C (permissive temperature) and 39°C (restrictive temperature) in Vero and HuH-7 cells. Cell monolayers in 96-well plates were inoculated with serial 10-fold dilutions of virus and replicate plates were incubated at 35 and 39°C for five days in temperature-controlled water baths. Virus replication was determined by immunoperoxidase staining as previously described (Blaney *et al.,* 2001). A collection of 193 5-FU virus clones demonstrated a 100-fold or greater reduction in titer at 39°C in either cell line, and these presumptive *ts* viruses were further characterized. The efficiency of plaque formation (EOP) at permissive and restrictive temperatures and the plaque size of each of the 193 virus clones were determined as follows. Serial 10-fold dilutions of virus suspension were inoculated onto confluent Vero cell and HuH-7 cell monolayers in replicate 24-well plates. After incubation at 35°C for 2 h, monolayers were overlaid with 0.8% methylcellulose (EM Science, Gibbstown, NJ) in L-15 medium (Quality Biologicals, Gaithersburg, MD) supplemented with 2% FBS, gentamicin, and L-glutamine. After incubation of replicate plates for 5 days at 35, 37, 38, or 39°C in temperature-controlled water baths, plaques were visualized by immunoperoxidase staining and counted as previously described. Plaque size of each of the 193 viruses was evaluated at the permissive temperature (35°C) and compared to that of DEN4 2A-13 parallel-passaged control virus with a wild-type plaque size. Mutant viruses incubated at the permissive temperature of 35°C which had a plaque size $\leq$1 or 0.4 mm (approximately $\leq$50% the size of wild-type DEN4 2A-13) in Vero or HuH-7 cells, respectively, were designated as having a *sp* phenotype. The level of temperature sensitivity and plaque size of each virus was confirmed in at least two separate experiments. Seventy-five viruses which were confirmed to have a putative *ts* and/or *sp* phenotype were biologically cloned an additional two times and phenotypes were reassessed. Twenty-two of the 75 terminally diluted viruses were found to have a *sp* phenotype. Sixteen of the 22 *sp* mutant viruses were also found to have a *ts* phenotype as defined by a 2.5 or 3.5 $\log_{10}$PFU/ml reduction in virus titer in Vero or HuH-7 cells, respectively, at restrictive temperature compared to the permissive temperature of 35°C, as previously described (Blaney *et al.,* 2001). Twenty of the 75 terminally diluted viruses were found to have a *ts* phenotype without a *sp* phenotype and were previously described (Blaney *et al.,* 2001). The remainder of the 75 viruses did not meet either criteria for a *ts* or *sp* mutant virus.

Evaluation of *sp* mutant viruses for restricted replication in suckling mice and SCID mice transplanted with HuH-7 cells

Animal experiments were carried out in accordance with the regulations and guidelines of the National Institutes of Health, Bethesda, MD. Growth of DEN4 5-FU mutant viruses was determined in Swiss Webster suckling mice (Taconic Farms, Germantown, NY). Groups of six seven-day-old mice were inoculated intracerebrally with 10^4 PFU of virus in 30 μl Opti-MEM I (Invitrogen) and the brain of each mouse was removed 5 days later and individually analyzed as previously described (Blaney *et al.,* 2001). Clarified supernatants of 10% suspensions of mouse brain were frozen at −70°C, and the virus titer was determined by plaque assay in Vero cells.

For analysis of DEN4 virus replication in SCID-HuH-7 mice, four- to six-week-old SCID mice (Tac:Icr:Ha(ICR)-*Prkdc*^{scid}) (Taconic Farms) were injected intraperitoneally with 10^7 HuH-7 cells suspended in 200 μl phosphate-buffered saline (PBS). For transplantation, HuH-7 cells were propagated in cell culture as described above and harvested by trypsinization at approximately 80% confluence. Cells were washed twice in PBS, counted, resuspended in an appropriate volume of PBS, and injected into mice. Tumors were detected in the peritoneum five to six weeks after transplantation, and only mice with apparent tumors were used for inoculation. Mice were infected by direct inoculation into the tumor with 10^4 PFU of virus in 50 μl Opti-MEM I. Mice were monitored daily for 7 days and serum for virus titration was obtained by tail-nicking on Day 6 and 7. Approximately 400 μl blood was collected in a serum separator tube (Sarstedt, Germany) and centrifuged, and serum was aliquoted and stored at −70°C. The virus titer was determined by plaque assay in Vero cells. Seven days after infection, most mice developed morbidity and all mice were sacrificed. Tumors were excised and weighed to confirm uniformity of the experimental groups.

Determination of the complete genomic sequence of the *sp* mutant viruses

The nucleotide sequence of the 5-FU-mutagenized DEN4 viruses was determined as described previously (Durbin *et al.,* 2001). Briefly, genomic RNA was isolated from virus clones and cDNA was prepared by reverse transcription and served as template for the generation of overlapping PCR fragments. A panel of primers was designed to sequence both strands of the PCR product from which consensus sequences were assembled and analyzed. The nucleotide sequence of the 5′ and 3′ regions of the virus genome was determined after circularization of the RNA genome as previously described (Durbin *et al.,* 2001).

Generation of recombinant DEN4 viruses

Cloning and transfection of full-length DEN4 cDNA clones were performed as previously described (Blaney *et al.,* 2001). Briefly, 500 to 2000 nucleotide fragments of the DEN4 cDNA clone, p4, were subcloned into a modified pUC119 vector and point mutations were introduced by site-directed mutagenesis and confirmed by sequence analysis. Full-length RNA transcripts were generated by *in vitro* transcription of the mutagenized p4 cDNA clones using the AmpliCap SP6 message maker kit (Epicentre Technologies, Madison, WI). C6/36 cells were transfected with RNA combined with DOTAP liposomal transfection reagent (Roche, Indianapolis, IN), and cell culture media were harvested 5 to 7 days later. Recovered viruses were terminally diluted twice and propagated in Vero cells. Recombinant DEN4 viruses are designated rDEN4—followed by the nucleotide position of the incorporated mutation.

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